Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 56–59, January 1983
Biochemistry

Molecular cloning of a cDNA specific for the murine p53 cellular tumor antigen

(monoclonal antibodies/polysome immunoselection/cDNA library hybrid selection)

MOSHE OREN* AND ARNOLD J. LEVINE†

*The Weizmann Institute of Science, Department of Chemical Immunology, Rehovot 76100, Israel; and †State University of New York, Department of Microbiology, School of Medicine, Stony Brook, New York 11794

Communicated by James E. Darnell, Jr., October 1, 1982

ABSTRACT **A cDNA library was constructed from sucrose-gradient-fractionated mRNA from SVT2, a mouse cell line transformed by simian virus 40. Polysomes containing the p53 messenger were specifically immunoprecipitated with monoclonal antibodies against the protein and used to prepare mRNA. This immunoselected mRNA, enriched 1,000- to 2,000-fold for p53-specific sequences, was used to make a cDNA probe and to screen the cDNA library. When approximately 10,000 colonies were screened by differential hybridization with probes made from immunoselected vs. nonenriched mRNA, a single clone was found that contained p53-specific sequences. The identity of this clone, termed pp53-208, was confirmed by the ability of its DNA to hybridize the mRNA coding for p53 (hybrid selection assay). When hybridized to a blot of *Eco*RI digested mouse DNA, the pp53-208 insert reacted with a single 3.3-kilobase band, suggesting that it is complementary to a single gene.**

Various transformed cells exhibit increased levels of a particular protein, termed the p53 cellular tumor antigen (for review see ref. 1). The precise function of the p53 protein is still unknown. However, the fact that low amounts of it are found in nontransformed growing cells (2–5), combined with the extremely short half-life of the protein in such cells (5), suggests that p53 may have some regulatory role during normal cell growth (6). It is thus conceivable that any mechanism leading to alterations in the level of p53 may result in the disruption of these regulatory processes and eventually lead to cell transformation.

Previous studies have indicated that the differential expression of p53 in normal and transformed cells can be brought about by more than a single mechanism. Thus, simian virus 40 (SV40) transformation of mouse cells results in a dramatic increase in the half-life of p53 protein, leading to its substantial accumulation without affecting the level of translatable p53 mRNA (2, 5). On the other hand, the difference in the amounts of p53 between undifferentiated and differentiated F9 teratocarcinoma cells can be totally accounted for by alterations in the level of the translatable mRNA (7). Similarly, protein stabilization may lead to p53 accumulation in cells transformed by adenovirus (8) and Epstein–Barr virus (9), whereas a transcriptional activation has been suggested in the case of induced lymphocytes (10).

Clearly, understanding the various mechanisms leading to increased p53 levels may shed light upon the relationship between this protein and transformation. Nevertheless, studies along this line were seriously limited by the lack of an appropriate nucleic acid probe. To overcome this difficulty, we attempted to prepare a cDNA clone of the p53 mRNA from SVT2 cells, an SV40-transformed mouse cell line. This report describes the isolation and identification of such a cDNA clone, designated pp53-208, and its initial characterization.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Construction of cDNA Library. Poly(A)$^+$ RNA from SVT2 cells was prepared and fractionated over a sucrose gradient as described (5). The fractions containing the highest proportion of p53 mRNA, as determined by *in vitro* translation (5), were utilized as templates for cDNA synthesis. The procedure employed was essentially that of Land *et al.* (11) except that prior to first-strand synthesis the mRNA was incubated at 37°C for 5 min in 5 mM methylmercury hydroxide. The average length of the poly(dC) homopolymer tails added to the double-stranded DNA was approximately 25 bases.

Plasmid pBR322 was digested with *Pst* I, and the fragments were chromatographed over a Bio-Gel A-50m column (Bio-Rad) and d(G) tailed to an average length of 25 bases. The tailed vector was further purified over a sucrose gradient as described by Norgard *et al.* (12). The cDNA was annealed with the vector in 10 mM Tris·HCl, pH 7.4/100 mM NaCl/0.2 mM EDTA. The mixture was heated to 65°C for 3 min, incubated at 48°C for 30 min and 42°C for 90 min, and then slowly allowed to cool to room temperature. Bacterial transformation was done with *Escherichia coli* strain RRI (12). The transformation efficiency was 1–3 × 10^7 transformants per μg of supercoiled vector. Transformant colonies were plated at a high density and their DNA was transferred to nitrocellulose membranes by the method of Thayer (13) and hybridized with appropriate probes.

Preparation of Hybridization Probes. SVT2 RNA was enriched for p53-specific sequences by the polysome immunoselection method, essentially as described previously (14), employing the monoclonal anti-p53 antibody RA32C2 (15). The RNA was then chromatographed over two consecutive oligo(dT)-cellulose columns, the second one consisting of 10 μl of the cellulose in a micropipettor tip. The RNA was eluted in 30 μl of water and used directly for cDNA synthesis. The reaction mixture contained 50 mM Tris·HCl at pH 8.3; 30 mM KCl; 9 mM $MgCl_2$; 5 mM dithiothreitol; dATP, dGTP, and dTTP at 1 mM each; 50 μCi of dCTP (1 Ci = 3.7 × 10^{10} becquerels); 10 units of avian myeloblastosis virus reverse transcriptase (generously provided by J. Beard, Life Sciences); and either 1.5 μg of oligo(dT) [$(dT)_{12-18}$, Boehringer Mannheim] or 0.4 μg of calf thymus primer, prepared by the method of Taylor *et al.* (16) (generous gift of A. Ullrich, Genentech). The reaction proceeded at 37°C for 3–5 hr. When immunoselected RNA made from 1.5 g of cells was used in such a reaction, the approximate yield was 3 × 10^7 cpm with calf thymus primer and 3 × 10^6 cpm with oligo(dT) primer. A probe complementary to total poly(A)$^+$

Abbreviations: p53, murine M_r 53,000 cellular tumor antigen; SV40, simian virus 40; NaCl/Cit, 0.15 M NaCl/0.015 M sodium citrate.

RNA was similarly made, except that the template was prepared by passing 20 μg of SVT2 cytoplasmic RNA over two consecutive oligo(dT)-cellulose columns.

Screening of cDNA Library. The cDNA probes were hybridized to colony blots at 67°C in a mixture of 5× NaCl/Cit (1× NaCl/Cit is 0.15 M NaCl/0.015 M sodium citrate), 50 mM sodium phosphate at pH 7.0, 20 mM sodium pyrophosphate, 5× Denhardt's solution (17), 0.2% $NaDodSO_4$, poly(adenylic acid) at 100 μg/ml, and denatured *E. coli* DNA at 100 μg/ml. Colonies exhibiting the predicted hybridization pattern were picked, reisolated, and retested by differential hybridization to cDNA probes.

Hybridization-Selection. Clones to be tested were grown in 250 ml of M9 medium and amplified in the presence of cytidine at 1 mg/ml (18). DNA was purified by isopycnic centrifugation in CsCl/ethidium bromide gradients. Fifteen micrograms of DNA was digested with *Eco*RI, heat denatured, and bound to a 1-cm^2 square of nitrocellulose membrane. The bound DNA was hybridized with 200 μg of total cytoplasmic SVT2 RNA at 45°C for 4 hr in the buffer described by Ricciardi *et al.* (19). The nitrocellulose membrane was consecutively incubated for 15 min at 45°C in the above hybridization buffer and 15 min at 60°C in 1× NaCl/Cit/0.2% $NaDodSO_4$ and then extensively washed on a Vortex mixer in the latter solution. After three washes at 60°C in 2 mM EDTA at pH 7.0, the bound RNA was eluted by heating to 90°C for 45 sec in water containing 3 μg of bovine liver tRNA and, finally, precipitated with ethanol. *In vitro* translation, immunoprecipitation, and protein analysis were as described (5).

Genomic DNA Analysis. High molecular weight mouse DNA (kindly provided by G. Rechavi) was digested with *Eco*RI, transferred to nitrocellulose sheets (20), and hybridized with a nick-translated insert derived by cleaving plasmid DNA with *Pst* I. Hybridization was at 42°C in a mixture containing 50% (vol/vol) formamide, 5× NaCl/Cit, 5× Denhardt's solution, 50 mM sodium phosphate at pH. 6.5, denatured *E. coli* DNA at 100 μg/ml, and 10% dextran sulfate for 24 hr. Hybridized filters were washed for 1 hr in 35% formamide/5× NaCl/Cit/5× Denhardt's solution/50 mM sodium phosphate, pH 6.5, at 42°C followed by several washes at 55°C in 3× NaCl/Cit/0.1% $NaDodSO_4$ and finally subjected to autoradiography.

RESULTS

Immunoselection of p53 mRNA. The screening of the cDNA library for the presence of p53-specific clones required a probe substantially enriched for p53 sequences. To achieve this goal, polysomes were prepared from SVT2 cells and immunoprecipitated with a monoclonal antibody against p53 (14). The RNA was isolated and chromatographed twice over oligo(dT)-cellulose, and a small aliquot of the RNA was assayed by *in vitro* translation. The data (Fig. 1) clearly demonstrated a significant enrichment for the p53 mRNA. In unselected SVT2 polysomal mRNA, the p53 messenger constitutes approximately 0.01% as estimated by *in vitro* translation (unpublished data). After immunoselection it becomes the major translatable species, the enrichment factor being 1,000–2,000. All the other translation products programmed by the immunoselected RNA correspond to abundant cellular messenger species (Fig. 1, lanes 1 and 3), probably resulting from nonspecific trapping.

Screening the cDNA Library for p53 cDNA Clones. A cDNA library was prepared from the 17.5S fraction of cytoplasmic SVT2 mRNA. The library was then screened by hybridization with a radioactive cDNA probe made with immunoselected SVT2 mRNA as a template. A substantial proportion of the bacterial colonies exhibited a positive reaction (data not shown).

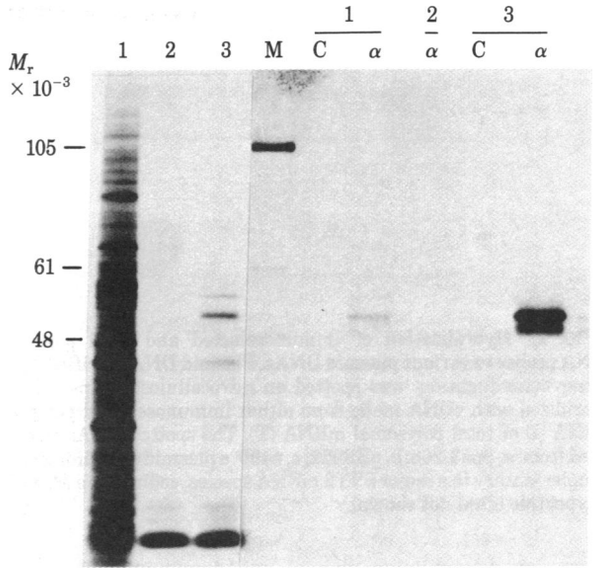

FIG. 1. Enrichment of SVT2 mRNA for p53-specific sequences by polysome immunoprecipitation. Polysomes were prepared from SVT2 cells and immunoprecipitated as described (14), employing the monoclonal anti-p53 antibody RA3-2C2 (15). The immunoselected RNA as well as total polysomal mRNA was translated in the reticulocyte lysate system. An aliquot of each translation reaction was applied directly to a $NaDodSO_4$/polyacrylamide gel, whereas the rest was first immunoprecipitated with anti-p53 monoclonal antibody L421 (21) and then electrophoresed. (*Left*) Lanes 1, 2, and 3, translation products made in the presence of total polysomal mRNA, in the absence of exogenous RNA, and in the presence of immunoselected polysomal RNA, respectively. (*Right*) Same samples, after incubation with either control serum (C) or monoclonal anti-p53 antibody L421 (α). M, molecular weight marker proteins from adenovirus-infected cells. Note that the lower M_r 50,000 band precipitated by the antibody is not present before the final immunoprecipitation (lane 3 vs. lane 3α) and thus is probably generated proteolytically from p53 during that step. *Right* was purposefully overexposed to allow detection of minor bands.

Because the immunoselected RNA contains substantial levels of contaminating abundant messenger species (Fig. 1, lane 3), it was conceivable that most positive colonies harbored cDNA derived from those abundant species. Therefore, a second cDNA probe was made with total cytoplasmic mRNA as a template, and it was hybridized to the same library. When a library of approximately 10,000 colonies was screened by this procedure, a single colony was found that reacted solely with the immunoselected probe and not with the total cytoplasmic probe. This clone, exhibiting the hybridization pattern expected of a p53-specific plasmid, was designated pp53-208.

Identification of the p53 cDNA Clone. To further characterize the candidate p53 clone, it was grown in a large culture and plasmid DNA was extracted. This DNA was first tested for differential hybridization to the two cDNA probes described in the previous section (Fig. 2). It can be seen that pp53-208 DNA exhibited the expected hybridization pattern, reacting strongly with the immunoselected probe (I) and practically not at all with the total cytoplasmic probe (T). To provide a more definitive identification, pp53-208 DNA was bound to a nitrocellulose and used in a hybridization selection assay. The bound RNA was eluted and translated *in vitro*, and the product was immunoprecipitated with a monoclonal antibody specific for the p53 protein. The result of this experiment is shown in Fig. 3. Whereas pBR322 DNA or DNA derived from pools of different cDNA clones failed to bind any p53 mRNA, pp53-208 DNA selected a substantial level of this messenger, indicating that it contains a p53 mRNA-derived insert. To further substantiate

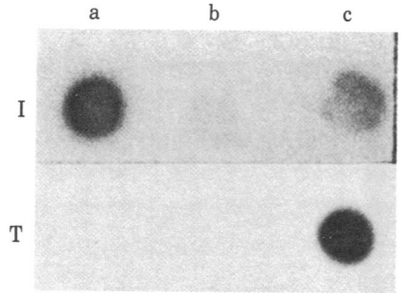

FIG. 2. Hybridization of immunoselected and total polysomal cDNA probes to various plasmid DNAs. Plasmid DNA, purified by isopycnic centrifugation, was spotted on nitrocellulose strips (22) and hybridized with cDNA made from either immunoselected polysomal mRNA (I) or total polysomal mRNA (T). The spotted DNAs were derived from: a, pp53-208; b, pBR322; c, p490, a plasmid containing cDNA complementary to a major SVT2 mRNA species, coding for a M_r 47,000 polypeptide (data not shown).

this conclusion, the insert was excised from pp53-208 DNA by *Pst* I digestion, labeled, and used to screen another 30,000 cDNA clones. Two additional clones were found that hybridized to the labeled probe. When assayed by hybridization-selection, both these clones strongly selected p53 mRNA, whereas many other clones from the same and from additional cDNA libraries

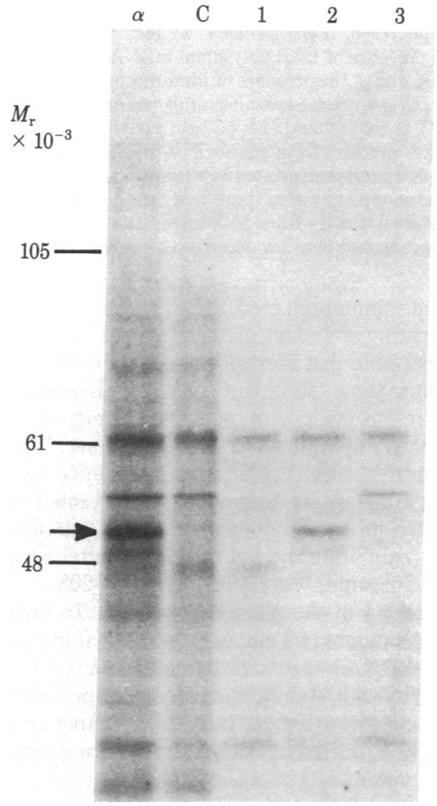

FIG. 3. Hybridization-selection analysis of cDNA clones. Plasmid DNA was bound to nitrocellulose and mRNA complementary to the insert was selected and characterized by *in vitro* translation. Lanes α and C, polypeptides detected after translation of total SVT2 mRNA and immunoprecipitation with anti-p53 monoclonal antibody or control serum, respectively. The arrow denotes the position of p53. Lanes 1, 2, and 3, polypeptides immunoprecipitated with anti-p53 monoclonal antibody after translation of mRNA selected by p490 (see Fig. 2) by pp53-208 and by a mixture of three other clones from the same cDNA library, respectively.

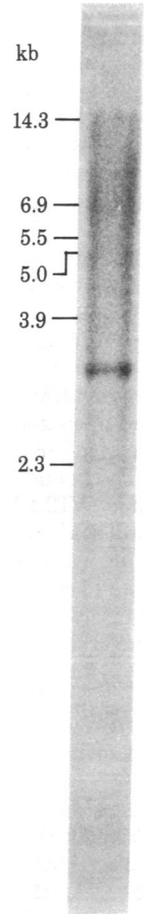

FIG. 4. Hybridization of pp53-208 to mouse genomic DNA. High molecular weight BALB/c DNA (20 μg) was digested to completion with *Eco*RI and transferred to nitrocellulose (20). pp53-208 DNA was digested with *Pst* I, and the cDNA insert was purified by gel electrophoresis and electroelution and then labeled by nick-translation (5×10^7 cpm/μg). The labeled probe was hybridized to the genomic blot. Numbers denote the positions and sizes [in kilobases (kb)] of markers coelectrophoresed on the gel (DNA of λ bacteriophage Charon 4A digested with *Bam*HI and *Eco*RI).

failed to bind any detectable amounts of this mRNA (data not shown).

Hybridization of pp53-208 to Mouse Genomic DNA. High molecular weight DNA was prepared from the liver of BALB/c mice, digested with *Eco*RI, blotted by the Southern procedure (20), and hybridized to a nick-translated pp53-208 cDNA insert. As seen in Fig. 4, only one band was revealed by this assay, suggesting that the cloned cDNA is complementary to a single mouse gene. When DNA from different mouse strains as well as from several murine tumor lines was similarly studied, the same 3.3-kilobase band was always found, and there was no indication of polymorphism (unpublished observations).

DISCUSSION

This report describes the derivation of pp53-208, a plasmid containing sequences present in the mRNA of the murine p53 cellular tumor antigen. The cloning was greatly facilitated by the ability to specifically immunoprecipitate p53-synthesizing polysomes with monoclonal antibodies and to obtain mRNA highly enriched for p53 messenger. A plasmid containing p53-specific sequences was isolated by differential hybridization with probes made from this enriched mRNA and from total polysomal mRNA. The identity of the cloned sequences in this plasmid was determined by hybridization-selection. In these experiments, both pp53-208 (Fig. 3) and two other plasmids sharing inserted sequences with it (data not shown) efficiently bound the p53 mRNA, whereas many other plasmids from several cDNA libraries (Fig. 3 and unpublished observations) repeatedly failed to do so. The size of the insert in pp53-208 is about 320 base pairs, including a total of 42 residues added during the homopolymer tailing steps (unpublished data). In theory, this

insert could be derived from a mRNA that does not code for p53 but happens to share extensive sequence homology with it within the cloned region. If that were the case, pp53-208 should be complementary to at least two genes in the mouse genome. However, as seen in Fig. 4, only a single band is detected when pp53-208 DNA is hybridized to a Southern genomic blot. Thus, it seems very unlikely that pp53-208 is derived from any mRNA but that coding for the p53 itself.

The criteria employed here to identify the pp53-208 clone as one containing nucleotide sequences complementary to authentic p53 mRNA rests upon hybrid selection of this mRNA and its *in vitro* translation. The p53 protein translated *in vitro* has been identified by the specificity of the antibody used in immunoprecipitation, by the molecular weight of the *in vitro* product (Figs. 1 and 3), and by peptide map analysis of the *in vitro* and *in vivo* p53 proteins (5). The fact that three independent cDNA clones with related sequences all select p53 mRNA in hybridization further substantiates these results. For these hybrid selection experiments the *in vitro* protein product was immunoprecipitated (Fig. 3) and analyzed on $NaDodSO_4$/polyacrylamide gels. When clone pp53-208 is employed to select mRNA from SV40-transformed cells, and this mRNA is translated *in vitro*, p53 is the only protein, of the total proteins made *in vitro*, whose mRNA is specifically selected by the hybridization procedure. Thus, immunoprecipitation of the protein made *in vitro* is not required to demonstrate the specificity of the p53 cDNA clone.

Increased amounts of p53 can be detected in many instances of cell proliferation and transformation (1). In several cases, alterations in mRNA levels appear to be involved in these processes (7, 10). The availability of a p53 cDNA clone now makes it possible to approach this issue in a more direct way as well as to screen diverse cells and tissues for overproduction of the p53 mRNA. The detection of changes in p53 gene expression and the definition of factors affecting those changes should help to determine more precisely the relationship between the p53 and cell transformation.

M.O. thanks Dr. D. Givol for providing facilities and for many helpful discussions and Dr. G. Rechavi for help with the genomic blotting. We are grateful to Drs. K. Segawa, R. Oren, and R. Robinson for help during the initial stages of this work and to C. Sullivan, A. K. Teresky, and T. Mangano for technical assistance. This work was supported in part by Grant CA28127-02 from the National Cancer Institute.

1. Klein, G., ed. (1982) *Advances in Viral Oncology* (Raven, New York), Vol. 2.
2. Linzer, D. I. H., Maltzman, W. & Levine, A. J. (1979) *Virology* **98**, 308–318.
3. Simmons, D. T. (1980) *J. Virol.* **36**, 519–525.
4. Dippold, D. G., Jay, G., DeLeo, A., Khoury, G. & Old, L. J. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 1695–1699.
5. Oren, M., Maltzman, W. & Levine, A. J. (1981) *Mol. Cell. Biol.* **1**, 101–110.
6. Campisi, J., Medrano, E. E., Morreo, G. & Pardee, A. B. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 436–440.
7. Oren, M., Reich, N. C. & Levine, A. J. (1982) *Mol. Cell Biol.* **2**, 443–449.
8. Sarnow, P., Shih, H. Y., Williams, J. & Levine, A. J. (1982) *Cell* **28**, 387–394.
9. Luka, J., Jornvall, H. & Klein, G. (1980) *J. Virol.* **35**, 592–602.
10. Milner, J. & Milner, S. (1981) *Virology* **112**, 785–788.
11. Land, H., Grez, M., Hauser, H., Lindenmaier, W. & Schutz, G. (1981) *Nucleic Acids Res.* **9**, 2251–2266.
12. Norgard, M. V., Tocci, M. J. & Monahan, J. J. (1980) *J. Biol. Chem.* **255**, 7665–7672.
13. Thayer, R. E. (1979) *Anal. Biochem.* **98**, 60–63.
14. Oren, M. & Levine, A. J. (1981) *Virology* **113**, 790–793.
15. Rotter, V., Witte, O. N., Coffman, R. & Baltimore, D. (1980) *J. Virol.* **36**, 547–555.
16. Taylor, J. M., Illmensee, R. & Summers, J. (1976) *Biochim. Biophys. Acta* **442**, 324–330.
17. Denhardt, D. T. (1966) *Biochem. Biophys. Res. Commun.* **23**, 641–646.
18. Norgard, M. V., Emigholz, K. & Monahan, J. J. (1979) *J. Bacteriol.* **138**, 270–273.
19. Ricciardi, R. P., Miller, J. S. & Roberts, B. E. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 4927–4931.
20. Southern, E. M. (1975) *J. Mol. Biol.* **98**, 503–517.
21. Harlow, E., Crawford, L. V., Pim, D. C. & Williamson, N. M. (1981) *J. Virol.* **39**, 861–869.
22. Schutzbank, T. E., Robinson, R., Oren, M. & Levine, A. J. (1982) *Cell* **30**, 481–490.